Chrna5 is a marker of acetylcholine super-responder subplate neurons with specialized expression of nicotinic modulator proteins

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Pages: 34
Figures: 9
Tables: 1
Supplementary materials: 5
Words: Abstract (150), Introduction (609), Discussion (1106)

Conflicts of interest: None

Acknowledgements: We acknowledge the generous support of the Canadian Institutes of Health Research (CIHR MOP 89825, EKL; CIHR PJT-153101, EKL), the Canada Research Chair in Developmental Cortical Physiology (EKL), an Ontario Graduate Scholarship (SV). We thank Ms. Janice McNabb and Mr. Ha-Seul Jeoung for expert technical assistance. Earlier presentations of this work received valuable feedback from Dr. Junchul Kim, Dr. Beverly Orser, and Dr. Steve Prescott of the University of Toronto.

Abstract (150 words)

Attention depends on cholinergic excitation of prefrontal neurons. Knockout/knockdown studies indicate nicotinic alpha5 subunits encoded by *Chrna5* are required for this response, but their native cellular roles and molecular interactions are unknown. Here, we probe endogenous cholinergic regulation of prefrontal *Chrna5*-expressing neurons (Chrna5+) using compound transgenic mice. Chrna5+ neurons show high sensitivity to acetylcholine, with a subpopulation clearly different from nearby, well-examined Syt6+ cells. Transcriptomic analysis reveals this distinct Chrna5+ population as subplate neurons, a diverse group of firstborn cells that have eluded previous transgenic characterization. Intriguingly, Chrna5+ subplate neurons express a distinct profile of GPI-anchored lynx prototoxins, suggesting specialized regulation of their cholinergic responses. In brain slices, endogenous nicotinic responses can be bidirectionally altered by perturbing GPI-anchored lynxes with phospholipase C activation or exogenous application of recombinant Ly6g6e prototoxin. Our work reveals cell-type specific *Chrna5* and Lynx modulation leading to exquisite cholinergic sensitivity of prefrontal subplate neurons in adulthood.

Teaser

Chrna5-expression identifies subplate neurons in the adult prefrontal cortex with enhanced cholinergic sensitivity.

Introduction (609 words)

Cholinergic modulation of the medial prefrontal cortex (mPFC) is essential for attention and detection of sensory cues (*1–4*). Deep-layer pyramidal neurons in the PFC are critically involved in such executive function (*5–7*) and are robustly excited by acetylcholine (*8*, *9*), through nicotinic and muscarinic receptor activation (*10*). The α5 nicotinic receptor subunit encoded by *Chrna5* is specifically expressed in deep-layer pyramidal neurons (*11*, *12*), forming high-affinity nicotinic receptors in combination with α4 and β2 subunits. Electrophysiological, behavioural, and genetic evidence in both rodents and humans point to an important role of *Chrna5* expression for nicotinic receptor function, attention, and executive function.

Constitutive knock out of *Chrna5* in mice or knockdown in the adult rat PFC disrupts attention and reduce nicotinic receptor activation by exogenous acetylcholine stimulation in layer 6 neurons (*13–15*). Optogenetic experiments in *Chrna5*$^{-/-}$ mice show that *Chrna5* is required for rapid onset of postsynaptic cholinergic activation and prevents desensitization of the endogenous cholinergic response during prolonged stimulation (*16*). In humans, the non-synonymous rs16969968 (D398N) polymorphism in *Chrna5* is associated with nicotine dependence, schizophrenia and cognitive impairment (*17–19*). Nicotinic α4β2α5 receptors with the D398N polymorphism have partial loss of function, attributed to changes in receptor desensitization, calcium permeability, or membrane trafficking (*20–22*).

Despite the clear indications of alpha5 nicotinic receptor involvement in attention and prefrontal cholinergic activation, systematic characterization of *Chrna5*-expressing neurons using modern genetic tools is lacking. Deep-layer neurons include diverse corticothalamic (L6CT), corticocortical, and layer 6b (L6b) populations (*23–25*). *Chrna5* is predicted to be expressed in L6CT neurons (*8*, *26*), which are usually identified by their expression of *Syt6*, a conserved L6CT neuronal marker (*27–29*). *Syt6*-Cre mice have been widely used to characterize prefrontal L6CT neurons and their cholinergic properties (*30–32*). However, it is unclear whether these are the same neurons expressing *Chrna5*. Characterization of *Chrna5*-expressing neurons has been limited by the lack of verified antibodies for the α5 subunit that could be used for post-hoc immunostaining. Previous BAC-transgenic mice labeling *Chrna5*-expressing neurons had altered expression of other genes in the tightly linked *Chrna5/a3/b4* gene cluster, limiting their use for functional examination (*33*). This issue was circumvented by disrupting the open reading frames of *Chrna3/b4* in the BAC transgene to generate a *Chrna5*-Cre mouse without misexpression artifacts (*34*).

Using compound transgenic mice, we first interrogated the response to optogenetic cholinergic stimulation and found stronger and faster responses in prefrontal *Chrna5*-expressing (Chrna5+) versus nonlabelled neurons. This prompted multi-approach characterization of Chrna5+ neurons versus better-defined *Syt6*-expressing (Syt6+) neurons. A large subset of Chrna5+ neurons were dissimilar to Syt6+ neurons, demonstrating higher affinity acetylcholine responses. Single-cell RNAseq analysis revealed the expression of several subplate markers (*Cplx3*, *Ctgf*, and *Lpar1*) in this Chrna5+ subset, identifying them as subplate neurons born early in development that are critical for establishing thalamocortical connectivity (*35–37*). Chrna5+ subplate neurons had a distinct expression pattern of GPI-anchored Lynx prototoxins with significantly enhanced

100 expression of *Ly6g6e*, *Lypd1*, and *Lypd6b,* together capable of exerting complex modulation of
101 nicotinic receptor properties (*38*, *39*). Consistently, removing GPI-anchored lynxes by PLC
102 activation altered optogenetic nicotinic response onset and amplitude. Exogenous application of
103 Ly6g6e prototoxin suppressed Syt6+ but not Chrna5+ nicotinic responses, revealing complex cell-
104 type specific Lynx-mediated regulation of nicotinic receptors.

105 Recent studies have focused on cell-type specific modulation of nicotinic receptors by
106 different Lynx prototoxins and the consequences for cortical development and cognition (*40–43*).
107 Here, we discovered endogenous Lynx modulation of nicotinic properties relevant for attention in
108 subplate neurons expressing *Chrna5*. These findings provide the first characterization of Chrna5+
109 neurons and the molecular determinants underlying their cholinergic properties, while identifying
110 *Chrna5* as a marker for subplate neurons with greater cholinergic responses.

111 Methods

112 *Animals*

113 *Syt6*-Cre$^{/+}$GCaMP6s$^{/+}$ and *Chrna5*-Cre$^{/+}$GCaMP6s$^{/+}$ mice used for calcium imaging were obtained
114 by crossing *Chrna5*-Cre (Gift from Dr. Eric Turner) and *Syt6*-Cre mice (*Syt6*-Cre KI148,
115 RRID:MMRRC 037416-UCD, (*44*)) respectively, with Ai96 mice (JAX: 024106). For
116 electrophysiological recordings of labeled Chrna5+ and Syt6+ neurons, we used *Chrna5*-
117 Cre$^{/+}$Ai14$^{/+}$, and *Syt6*-Cre$^{/+}$Ai14$^{/+}$ mice respectively. *Syt6*-EGFP$^{/+}$ mice were additionally used for
118 few experiments (*Syt6*-EGFP EL71, RRID:MMRRC 010557-UCD, (*45*)).

119 Triple transgenic mice labeling both *Chrna5* and *Syt6*-expressing neurons with EGFP in
120 Syt6+ neurons and tdTomato in Chrna5+ neurons were used to examine the overlap between the
121 two cell types. *Syt6*-EGFP and Ai14 mice (*46*) were bred together and the offspring were crossed
122 with *Chrna5*-Cre mice to generate *Chrna5*-Cre$^{/+}$Ai14$^{/+}$*Syt6*-EGFP$^{/+}$ mice used for these
123 experiments. A set of experiments measuring optogenetic cholinergic responses was also
124 performed in ChAT-ChR2 (ChAT$^{/+}$) mice (JAX: 014546). To examine optogenetic cholinergic
125 responses in labeled Chrna5 and Syt6 cell populations, the respective Cre lines were crossed with
126 ChAT$^{/+}$Ai14$^{/+}$ mice to generate *Chrna5*-Cre$^{/+}$Ai14$^{/+}$ChAT$^{/+}$ and *Syt6*-Cre$^{/+}$Ai14$^{/+}$ChAT$^{/+}$ mice.

127 All animals were bred on a C57BL/6 background, except *Syt6*-EGFP which were Black
128 Swiss. Adult male and female animals age >P60 were used in the study. Mice were separated based
129 on sex after weaning at P21 and group-housed (2-4 mice per cage). Animals had ad libitum access
130 to food and water and were on a 12-h light/dark cycle with lights on at 7 AM. Guidelines of the
131 Canadian Council on Animal Care were followed, and all experimental procedures were approved
132 by the Faculty of Medicine Animal Care Committee at the University of Toronto. 42 mice were
133 used for the entire study, with similar numbers of males and females.

134 *Brain slicing and electrophysiology*

135 Slicing and electrophysiology followed procedures described previously (*16*). An intraperitoneal
136 injection of chloral hydrate (400 mg/kg) was given to anesthetize mice prior to decapitation. The
137 brain was rapidly extracted in ice cold sucrose ACSF (254 mM sucrose, 10 mM D-glucose, 26
138 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 3 mM KCl and 1.25 mM NaH_2PO_4). 400 μm thick

139 coronal slices of prefrontal cortex (Bregma 2.2 - 1.1) were obtained on a Dosaka linear slicer
140 (SciMedia, Costa Mesa, CA, USA). Slices were left to recover for at least 2 hours in oxygenated
141 (95% O_2, 5% CO_2) ACSF (128 mM NaCl, 10 mM D-glucose, 26 Mm $NaHCO_3$, 2 mM $CaCl_2$, 2
142 mM $MgSO_4$, 3 Mm KCl, and 1.25 mM NaH_2PO_4) at 30°C before being used for electrophysiology
143 or two-photon calcium imaging. Brain slices were transferred to the stage of a BX51WI
144 microscope (Olympus, Tokyo, Japan) and perfused with oxygenated ACSF at 30°C. Recording
145 electrodes (2 - 4 MΩ) containing patch solution (120 mM potassium gluconate, 5 mM KCl, 10
146 mM HEPES, 2 mM $MgCl_2$, 4 mM K_2-ATP, 0.4 mM Na_2-GTP and 10 mM sodium
147 phosphocreatine, pH adjusted to 7.3 using KOH) were used to patch pyramidal neurons in layer 6
148 - 6b based on morphology and proximity to white matter. Only regular spiking neurons were
149 included. Multiclamp 700B amplifier at 20 kHz with Digidata 1440A and pClamp 10.7 software
150 (Molecular devices) were used for data acquisition. All recordings were compensated for liquid
151 junction potential (14 mV). Voltage-clamp responses were examined at -75 mV and in current-
152 clamp at rest or starting from -70 mV.

153 ***Optogenetics***

154 5 ms pulses of blue light (473 nm) were delivered through the 60X objective lens with an LED
155 (Thorlabs, 2 mW) to excite channelrhodopsin containing cholinergic fibers. Pattern of stimulation
156 was as in a previous study, with 8 pulses of light delivered in a frequency accommodating manner
157 (*16*).

158 ***Pharmacology***

159 Acetylcholine (1mM, Sigma) was used to exogenously stimulate cholinergic receptors. Atropine
160 (200 nM, Sigma) and Dihydro-β-erythroidine (DHBE, 10 μM, Tocris) were used to competitively
161 block muscarinic receptors and β2 subunit-containing nicotinic receptors respectively.
162 Mecamylamine (5 μM, Tocris) was used to further non-competitively block nicotinic receptors.
163 Phospholipase C activator m-3M3FBS (25 μM, Tocris) was used to cleave GPI-anchored Lynx
164 prototoxins and the inactive ortholog o-3M3FBS (25 μM, Tocris) was used as a control (*47*).
165 Cyclodextrin (1 mM, Tocris) was included in a small subset of experiments to improve solubility
166 of 3M3FBS compounds, but no further improvement in efficacy was observed. Water soluble
167 recombinant Ly6g6e (0.5 mg/ml) was obtained by custom purification (Creative Biomart) and
168 used for exogenous application at 1: 1000 and 3:1000 dilution. Effects on nicotinic receptors were
169 not distinguishable between the two different protein concentrations. Only freshly thawed protein
170 aliquots were used for experiments.

171 ***Two-photon imaging***

172 Two-photon imaging of GCaMP6s calcium signals in L6 neurons was performed using a 60×
173 water-immersion objective with 0.90 numerical aperture using an Olympus Fluoview FV1000
174 microscope and a Titanium-Sapphire laser sapphire laser (Newport) at 930nm. Images were
175 sampled at 512 x 512 pixels (2.4 pixels/μm) at a frame rate of 0.9 Hz. Following a 2-minute
176 washout period for this initial application, GCaMP6s calcium signals were measured in response
177 to acetylcholine (1 mM, 15 s). The cellular responses to acetylcholine were measured at baseline,
178 then after application of competitive nicotinic receptor antagonist DHBE (10 μM, 10 min), and
179 again after the addition of muscarinic antagonist atropine (200 nM, 10 min).

Dual color two-photon imaging (910 nm excitation, using 570 nm dichroic mirror with green (540-595 nm) and red (570-620 nm) filters) was performed in brain slices of triple transgenic *Chrna5*-Cre$^{/+}$Ai14$^{/+}$*Syt6*-EGFP$^{/+}$ mice to examine overlap in fluorescent reporter expression between Chrna5+ and Syt6+ neurons. Z-stacks of 30 frames acquired in 1-µm steps were taken in layer 6 of mPFC slices and the maximum projection used to count cells with the cell counter feature in Fiji. A set of mPFC brain slices from *Chrna5*-Cre$^{/+}$Ai14$^{/+}$*Syt6*-EGFP$^{/+}$ mice were also fixed and mounted for confocal imaging with LSM880 (Leica) microscope.

Single cell RNAseq analysis

Single cell RNAseq data for Anterior Cingulate Cortex (ACA) of adult mice was taken from the ACA and MOP Smart-Seq (2018) database, with cell-type annotations from Whole cortex & Hippocampus Smart-Seq (2019) database from the Allen Institute for Brain Science at https://portal.brain-map.org/atlases-and-data/rnaseq (*48*, *49*). Single cell analysis was performed using the R package Seurat (v 4.04). Layer 5 and 6 glutamatergic neurons were selected and sorted into three cell classes based on their expression of *Chrna5* and *Syt6* genes: those expressing only *Chrna5* (Chrna5+, n = 243), only *Syt6* (Syt6+, n = 834), or both *Chrna5* and *Syt6* (Chrna5+Syt6+, n = 564). Expression (copies per million) greater than zero was used as the threshold. 781 cells did not express either *Chrna5* or *Syt6* and were not used in subsequent analyses. Rare cell types with fewer than 10 cells per group are not shown in the heatmap in figure 5 but are included for the differential expression analysis. The FindMarkers function in Seurat was used to identify genes differentially expressed between the Chrna5+ and Chrna5+Syt6+ populations. Adjusted p value < 0.05 was used as the cutoff for identifying differentialy expressed genes.

Analysis and statistics

Analysis of electrophysiological data was conducted on Clampfit 10.7 and Axograph. Rising slope was measured by fitting a line to the first 50ms of the optogenetic cholinergic responses. Cholinergic response magnitude in voltage clamp was determined by peak current (picoamperes) and charge transfer (picocoulombs) measured by the area under the current response for 1 second.

GCaMP6s imaging data were extracted using the multi-measure feature in Fiji. Maximum projections across time for each experiment were first used to identify acetylcholine-responsive cells and add them to the ROI manager, then fluorescence intensity at all timepoints for each cell was measured. Fluorescence was normalized to the background fluorescence averaged over the first 10 frames. Area under the peak (AUP) of the signal after baseline correction was used to quantify the magnitude of cells' response to acetylcholine. Percentage response remaining after DHBE and DHBE+Atropine was calculated from the cell AUP before and after the blockers.

GraphPad Prism 8 was used for statistical analysis and plotting graphs. Bar graphs depicting mean with standard error and boxplots with median and quartiles are shown. Effect sizes are reported as Cohen's d for major results (*50*). Unpaired t-tests or Mann-Whitney tests were used when comparing response properties between cell types, and paired t-test or Wilcoxon test to quantify effect of pharmacological manipulations within cells. Kolmogorov-Smirnov and Fisher's exact tests were used to compare cumulative distributions and proportions of cells respectively.

219 **Results**

220 ***Chrna5 expression identifies a distinct population of 'acetylcholine super-responder' neurons***
221 ***with stronger and faster-onset optogenetic cholinergic responses***

222 *Chrna5* is expressed in deep-layer pyramidal neurons in the prefrontal cortex and thought to
223 modulate their nicotinic receptor properties to improve attentional processing. However,
224 characterization of prefrontal *Chrna5*-expressing (Chrna5+) neurons has not been undertaken
225 previously due to a lack of viable labeling strategies. Using recently available *Chrna5*-Cre mice
226 (*34*), we generated triple transgenic *Chrna5*-Cre$^{/+}$Ai14$^{/+}$ChAT-ChR2$^{/+}$ mice (**Fig 1A**) to interrogate
227 optogenetic cholinergic responses in labeled Chrna5+ neurons. These mice expressed tdTomato in
228 Chrna5+ neurons and channelrhodopsin 2 in cholinergic axons as seen by two-photon imaging
229 (**Fig 1B**). We recorded endogenous responses to optogenetic cholinergic stimulation by patch-
230 clamp electrophysiology in mPFC slices and compared the properties of labeled Chrna5+ and
231 unlabeled Chrna5- pyramidal neurons in layer 6 (**Fig 1C**). Neurons identified by their *Chrna5*
232 expression showed larger-amplitude cholinergic responses with significantly faster onset
233 compared to other unlabeled deep-layer pyramidal neurons (**Fig 1D-F**). The rising slope was
234 significantly larger in Chrna5+ neurons (220 ± 32 pA/s, 24 cells from 4 mice) compared to
235 unlabeled neurons (123 ± 22 pA/s, 15 cells; $t_{(37)} = 2.19$, $P = 0.02$; Cohen's d: 0.72). Similarly, peak
236 current evoked by optogenetic acetylcholine release was greater in Chrna5+ neurons (15 ± 2 pA)
237 compared to unlabeled neurons (8 ± 1 pA, $t_{(37)} = 2.52$, $P = 0.02$; Cohen's d: 0.83), as well as the
238 area of the cholinergic response ($t_{(37)} = 2.06$, $P = 0.046$). Intrinsic electrophysiological properties
239 including resting membrane potential, input resistance, capacitance, spike threshold, amplitude
240 and rheobase did not differ between Chrna5+ and unlabeled neurons (**Supplementary table 1**).
241 As reported previously in a broader layer 6 population (*16*), cholinergic responses in Chrna5+
242 neurons were under presynaptic muscarinic autoinhibitory control, which was relieved by atropine
243 resulting in even larger responses (94 ± 42% increase). The addition of nicotinic antagonist DHBE
244 eliminated optogenetic cholinergic responses (99 ± 3 % reduction), indicating the predominant
245 contribution of β2 subunit-containing nicotinic receptors in Chrna5+ neurons. Our initial
246 characterization of Chrna5+ neurons revealed them to be acetylcholine 'super-responders' with
247 stronger and faster onset cholinergic responses distinct from other deep-layer pyramidal
248 neurons.This prompted us to question how these cells differ from other well-defined layer 6
249 neurons labeled by *Syt6* expression (Syt6+).

250 *Syt6* has been extensively used as a marker to characterize layer 6 corticothalamic neurons
251 and their function during PFC-dependent tasks (*28*, *30*, *31*, *51*). Yet, the extent of overlap between
252 *Chrna5* and *Syt6* expressing deep-layer pyramidal neuron populations is unclear. Therefore, we
253 adopted an imaging strategy to visualize the distribution of Chrna5+ and Syt6+ neurons in the PFC
254 and determine the exact proportion of distinctive non-overlapping Chrna5+ neurons. We generated
255 a compound transgenic *Chrna5*-Cre$^{/+}$Ai14$^{/+}$*Syt6*-EGFP$^{/+}$ mouse to simultaneously express
256 tdTomato in Chrna5+ neurons and EGFP in Syt6+ neurons and performed confocal and two photon
257 imaging of the endogenous fluorescence of these reporters in mPFC brain slices. Chrna5+ and
258 Syt6+ neurons were both present primarily in layer 6, with a few Chrna5+ neurons in layer 5 (**Fig**
259 **1G, I**). Closer investigation confirmed the existence of a substantial proportion of exclusively

Chrna5+ neurons (37% of all labeled cells) which do not express *Syt6*, in addition to overlapping Chrna5+Syt6+ neurons (39%) which express both markers, and exclusively Syt6+ neurons (24%) which do not express *Chrna5* (N = 4 mice, **Fig 1H, J**). Thus, nearly half of all *Chrna5*-expressing neurons are not labeled by *Syt6*-expression and would be excluded in previous studies using *Syt6*-Cre mice.

Calcium imaging in Chrna5+ and Syt6+ layer 6 populations reveal a distinct subset of Chrna5+ neurons with highly resilient nicotinic receptor-mediated responses.

We next examined population level cholinergic responses in Chrna5+ and Syt6+ neurons to identify properties that distinguish the distinct subset of Chrna5+ neurons not found with Syt6-labeling approaches. We measured acetylcholine-evoked signals in multiple neurons simultaneously using *ex vivo* GCaMP6s calcium imaging in brain slices. We generated transgenic mice expressing GCaMP6s in either Chrna5+ (*Chrna5*-Cre Ai96) or Syt6+ (*Syt6*-Cre Ai96) neurons and performed two-photon imaging of mPFC layer 6 (**Fig 2A**). Calcium signals evoked by acetylcholine (1 mM, 15 s) were measured in Chrna5+ (supplementary video 1) and Syt6+ neurons (supplementary video 2). Changes in the calcium signal and proportions of acetylcholine-responsive neurons were measured after the application of competitive nicotinic receptor antagonist DHBE and addition of muscarinic antagonist atropine (**Fig 2A, B**). The proportion of acetylcholine-evoked calcium signals remaining after the application of the competitive nicotinic antagonist DHBE (10 µM, 10 min) was significantly greater in Chrna5+ neurons (35 ± 3 % of baseline, n = 71 cells, 6 mice; Fig 2C i), compared to Syt6+ neurons (21 ± 3% of baseline, n = 112 cells, 7 mice; Mann Whitney U = 2400, $P < 10^{-4}$). The cumulative distribution of responses remaining after DHBE was significantly right shifted in Chrna5+ neurons compared to Syt6+ neurons (Fig 2C ii, Kolmogorov Smirnov D = 0.37, $P < 10^{-4}$). A majority of Chrna5+ neurons (~83%) still showed acetylcholine-evoked responses after DHBE, whereas only fewer Syt6+ neurons (~50%) retained their responses, with a complete elimination of acetylcholine-evoked responses in the rest (Fig 2C iii, Fisher's exact test: $P < 10^{-4}$). Yet, the addition of muscarinic antagonist atropine did not attenuate the striking differences between Chrna5+ and Syt6+ neurons, raising the possibility of an underlying nicotinic mechanism (**Fig 2D**). In the presence of DHBE+ atropine, a subset of Chrna5+ neurons still showed substantial acetylcholine-evoked calcium signals (6 ± 1 % of baseline; Fig 2Di), whereas almost all Syt6+ neurons' responses were completely blocked (0.2 ± 0.1 % of baseline; Mann Whitney U = 2083, $P < 10^{-4}$) as seen from the cumulative distribution (Fig 2D ii, Kolmogorov Smirnov D = 0.36, $P < 10^{-4}$). The proportion of Chrna5+ and Syt6+ neurons showing acetylcholine-evoked responses after DHBE+ Atropine was significantly different (41% vs 6%, Fisher's exact test: $P < 10^{-4}$, **Fig 2D iii**).

While optogenetic cholinergic responses in Chrna5+ neurons were eliminated by the combination of DHBE and atropine, residual responses to strong stimulation with exogenous acetylcholine were observed in a large Chrna5+ subset following DHBE and atropine. Since DHBE is a competitive antagonist, it can be outcompeted by exogenous acetylcholine at high affinity nicotinic receptors. We hypothesized that the resilience of exogenous acetylcholine-evoked responses to competitive nicotinic receptor block in a large subset of Chrna5+ neurons indicated high affinity nicotinic receptors which would require non-competitive blockers to be completely blocked. We switched

301 to patch-clamp electrophysiology to test this hypothesis and investigate acetylcholine-evoked
302 spiking in individual Chrna5+ and Syt6+ neurons. We recorded current clamp responses to
303 acetylcholine (1 mM, 15 s) in labeled Chrna5+ and Syt6+ neurons from *Chrna5*-Cre$^{/+}$Ai14$^{/+}$ and
304 *Syt6*-Cre$^{/+}$Ai14$^{/+}$ or *Syt6*-EGFP mice respectively (**Fig 2E**). Chrna5+ neurons showed stronger
305 acetylcholine-evoked firing: attaining significantly higher peak firing frequency (29 ± 6 Hz, n=12
306 cells, 6 mice; $t_{(24)} = 2.74$, $P = 0.01$; Cohen's d: 1.08) compared to Syt6+ neurons (13 ± 2 Hz, n =
307 14 cells, 5 mice; Fig 3B). The intrinsic electrophysiological properties of Chrna5+ and Syt6+
308 neurons did not show statistically significant differences (**Supplementary table 2**). We next
309 examined the sensitivity of acetylcholine-evoked firing to competitive nicotinic receptor block by
310 DHBE in the presence of atropine. Acetylcholine-evoked firing was completely eliminated in all
311 Syt6+ neurons (**Fig 2F**) whereas a large subset of Chrna5+ neurons (7/11) retained their ability to
312 respond to acetylcholine (Average peak firing rate: 6 ± 2 Hz; $t_{(19)} = 3.22$, $P = 0.004$, unpaired t-
313 test) demonstrating similar resilience to competitive nicotinic receptor block as observed with
314 calcium imaging. We used the non-competitive nicotinic receptor blocker mecamylamine (*54*, *55*)
315 to test our hypothesis that nicotinic receptors in this Chrna5+ subset were higher affinity and
316 therefore allowed exogenous acetylcholine to outcompete DHBE. The addition of 5 μM
317 mecamylamine was sufficient to eliminate acetylcholine-evoked firing in all the Chrna5+ neurons
318 that were resilient to competitive nicotinic block ($t_{(4)} = 5.14$, $P = 0.007$, paired t-test; Fig 2F).
319 Together, our calcium imaging and electrophysiology experiments neurons revealed the existence
320 of a distinct subset of Chrna5+ neurons that were dissimilar to Syt6+ neurons, with high affinity
321 nicotinic responses that were resilient to competitive nicotinic antagonism. To answer why
322 enhanced cholinergic responses are found only in the Chrna5+ subset that is distinct from Syt6+
323 neurons, we turned to single-cell RNAseq to understand the diversity of Chrna5+ neurons and the
324 molecular determinants of their cholinergic properties.

325 ***Single-cell transcriptomics identifies Chrna5+ subplate neurons with distinct expression pattern***
326 ***of Lynx genes modulating cholinergic function***

327 We sought to identify whether the exclusive Chrna5+ neurons arise from a different set of cell
328 populations than Syt6+ neurons. Therefore, we extracted gene expression data of L5-6
329 glutamatergic neurons (n = 2422 cells) in the mouse anterior cingulate cortex from the Allen
330 Institute single cell RNAseq databases (*48*, *49*, *56*). We classified these deep-layer pyramidal
331 neurons into 3 groups as seen with imaging: those expressing only *Chrna5* (Chrna5+, n= 243),
332 both *Chrna5* and *Syt6* (Chrna5+ Syt6+, n = 834), or only *Syt6* (Syt6+, n = 564) **(Fig 3A)**. 781 cells
333 showed no expression of *Chrna5* or *Syt6* and consisted primarily of L6 Intratelencephalic cells
334 which have been previously shown to have purely muscarinic M2/M4 mediated hyperpolarizing
335 cholinergic responses (*16*, *57*). We focused on the Chrna5+, Syt6+ and Chrna5+Syt6+ groups to
336 examine their transcriptomic differences. Single-cell analysis revealed that the Chrna5+ group
337 primarily included L6b (44%), L5 near-projecting (L5NP, 19%), and L6CT neurons (30%),
338 whereas the Chrna5+Syt6+ and Syt6+ groups were predominantly composed of L6CT neurons
339 (>90%) **(Fig 3B)**. We examined the expression of marker genes in these respective groups to
340 validate our cell-classification. To our surprise, Chrna5+ neurons showed distinctive expression
341 of several marker genes- *Ctgf, Cplx3, Kcnab1, Lpar1* **(Fig 3B,C)** associated with subplate neurons
342 (*35*, *58*). Subplate neurons are vital for early brain development and L6b neurons are considered

343 descendants of this early-born population (*36*, *37*, *59*). Notably, the highest fold enrichment among
344 all differentially expressed genes in Chrna5+ neurons was found for subplate markers *Ctgf* (Fold
345 change, 5.69) and *Cplx3* (3.81) (**Table 1)**. Overall, Chrna5+ neurons including both L5NP and
346 L6b subpopulations highly express subplate marker genes. In contrast, *Syt6*-expressing
347 Chrna5+Syt6+ and Syt6+ groups are only enriched in the corticothalamic markers *Foxp2* and *Syt6*,
348 consistent with their corticothalamic subtype. These results support our imaging,
349 electrophysiological, and pharmacological results suggesting the exclusive Chrna5+ population is
350 distinct from typical L6CT *Syt6*-expressing neurons.

351 Next, we examined differential expression of genes with known effects on postsynaptic
352 cholinergic responses to identify molecular changes predictive of Chrna5+ 'super-responders' with
353 high-affinity nicotinic responses (**Fig 3D-E**). We selected cholinergic receptor genes (nicotinic
354 *Chrna5-2, Chrna7, Chrnb2-4,* and muscarinic subunits *Chrm1-4*), acetylcholinesterase (*Ache*),
355 and members of the family of genes that encode lynx proteins (*Ly6e, Ly6h, Ly6g6e, Lynx1, Lypd1,*
356 *Lypd6, Lypd6b*) known to allosterically modulate nicotinic receptor responses (*38*). We found
357 substantial and highly significant changes primarily in the expression of three lynx prototoxins
358 *Lypd1*, *Ly6g6e* and *Lypd6b* **(Fig 3D)**. While both Chrna5+ and Chrna5+Syt6+ populations express
359 *Chrna5,* there was slightly higher expression of *Chrna5* (25% increase) as well as lower expression
360 of the inhibitory muscarinic receptor *Chrm2* (20% decrease) in Chrna5+Syt6+ neurons. There
361 were no significant differences in other nicotinic and muscarinic subunit expression between the
362 two groups. Acetylcholinesterase, the enzyme that breaks down acetylcholine was highly
363 expressed (50% increase) in Chrna5+ neurons, which may benefit their nicotinic responses by
364 protecting receptors from overactivation and desensitization. The fold-change of the genes in
365 figure 3E between Chrna5+ and Chrna5+Syt6+ neurons is shown in **Supplementary table 3**.

366 Notably, the top three genes with highest fold change in Chrna5+ neurons were the GPI-
367 anchored lynx prototoxins: Lynx2 encoded by *Lypd1* (Fold change: 2.55), *Ly6g6e* (2.03), and
368 *Lypd6b* (1.51). Ly6g6e is thought to potentiate nicotinic receptors and slows nicotinic receptor
369 desensitization (*39*) which might contribute to high-affinity nicotinic responses in Chrna5+
370 neurons. Lypd6b has unknown effects (*60*) while Lynx2 is thought to inhibit α4β2 nicotinic
371 receptors (*61*). Lynx2 may also intracellularly suppress expression of lower affinity α4β2 receptors
372 (*39*) indirectly promoting the preferential expression of α5 containing high affinity nicotinic
373 receptors in Chrna5+ neurons. However, other lynxes such as Lynx1 which negatively modulates
374 α4β2 receptors (*41*) were expressed at similar levels in the majority of cells across all three groups.
375 The distinct pattern of expression of specific lynxes in Chrna5+ neurons suggests unexpectedly
376 complex endogenous control of nicotinic responses in these prefrontal subplate neurons.

377 ***Cell type specific modulation of optogenetic nicotinic responses by endogenous GPI-anchored***
378 ***lynxes***

379 Our transcriptomic analyses suggest that cells capable of fast and strong responses to optogenetic
380 release of endogenous acetylcholine contain complex molecular machinery to regulate these
381 responses. It is possible that this regulation endows deep layer prefrontal neurons with greater
382 dynamic range in responding to acetylcholine. To examine whether this transcriptomic prediction
383 is accurate, we sought to experimentally perturb endogenous lynx modulation. Members of the

lynx-family are GPI-anchored (**Fig 4A**), and work in cell expression systems (*39*) suggests these anchors can be cleaved via activation of phospholipase C (PLC). The potential impact of such GPI cleavage on nicotinic responses in a native system is not well understood. Perturbing lynx-mediated control could affect endogenous nicotinic properties in a complex manner (**Fig 4A**) since both positive (eg. Ly6g6e) and negative modulatory lynxes (eg. Lynx1) are expressed. To cleave GPI-anchored proteins, we used the PLC activator compound m-3M3FBS (*47*, *62–64*). Nicotinic responses of deep layer pyramidal neurons from ChAT-ChR2 mice to optogenetic acetylcholine release were recorded in the continuous presence of atropine before and after treatment with m-3M3FBS (25μM, 5 min; **Fig 4B**). The rising slope of the nicotinic responses showed a significant increase after m-3M3FBS treatment (23 ± 17%; Paired Cohen's d = 0.83; P = 0.008, Wilcoxon matched-pairs test), compared to the baseline change observed in the same cells prior to PLC activation (-6 ± 4%; 8 cells, 6 mice; **Fig 4C**). This increase was not observed with the inactive ortholog o-3M3FBS that does not activate PLC (Paired Cohen's d = 0.09, P = 0.625, Wilcoxon matched-pairs test, data not shown). The area under the nicotinic response known as charge transfer also showed a statistically significant increase following PLC activation (22 ± 7%; Cohen's d = 1.68; P = 0.016, Wilcoxon matched-pairs test; **Fig 4D**), compared to baseline change (-9 ± 6%). Thus, PLC activation causes a specific increase in nicotinic receptor responses, presumably due to cleavage of inhibitory GPI-anchored lynxes such as Lynx1. This result confirmed endogenous lynx modulation of optogenetic cholinergic responses in intact brain slices and validated our transcriptomic prediction of strong endogenous lynx regulation of nicotinic receptors in deep layer neurons.

Our transcriptomics results (Fig 3D) predicted that cell-type specific differences in Lynx modulation with enhanced expression of *Lypd1*, *Ly6g6e* and *Lypd6b* prototoxins in Chrna5+ neurons lead to the different cholinergic properties of *Chrna5* versus *Syt6*-expressing neurons. To validate this prediction experimentally, we tested whether exogenous application of a specific lynx prototoxin exerts differential effects in *Chrna5* and *Syt6*-expressing neurons. Prior work suggests the direction of these effects may be difficult to predict, however, since soluble and endogenous GPI-anchored prototoxins have different effects on nicotinic receptors (*41*). Since previous work predicts a substantial modulatory role for endogenous Ly6g6e, we obtained purified water-soluble recombinant Ly6g6e protein and examined its effects on optogenetic nicotinic responses in labeled Chrna5+ and Syt6+ neurons (**Fig 4F**). These experiments were conducted in *Chrna5*-Cre$^{/+}$Ai14$^{/+}$ChAT-ChR2$^{/+}$ and *Syt6*-Cre$^{/+}$Ai14$^{/+}$ChAT-ChR2$^{/+}$ mice. We hypothesized that the modulation of Chrna5+ neuronal nicotinic receptors by endogenous Ly6g6e would occlude the effect of exogenous soluble Ly6g6e, whereas Syt6+ neurons would be altered by exposure to the exogenous Ly6g6e (Fig 4G). Consistently, we found that 10 minute application of soluble Ly6g6e did not significantly alter the amplitude of optogenetically evoked nicotinic responses in labeled Chrna5+ neurons (Change in peak = -2.1 ± 1.2 pA, $t_{(6)}$ = 1.79, P = 0.12, paired t-test). However, in labeled Syt6+ neurons lacking endogenous expression of *Ly6g6e*, exogenous application of soluble Ly6g6e caused a significant decrease in the amplitude (Change in peak = -10 ± 1.8 pA, $t_{(8)}$ = 5.60, P < 0.001, paired t-test; **Fig 4H**). The change in peak and area of the nicotinic responses caused by solube Ly6g6e was significantly different between Chrna5+ and Syt6+ neurons (change in peak : $t_{(14)}$ = 3.43, P = 0.004; Change in area: $t_{(14)}$ = 2.53, P = 0.024, Unpaired t test; **Fig 4I - J**).

Our work examines the effects of GPI-anchored lynx prototoxins on native nicotinic receptor-mediated optogenetic responses, advancing from work in heterologous expression systems. These results are a first step in showing how endogenous lynx regulation of nicotinic

430 responses can act in a complex cell-type specific fashion leading to specialized cholinergic
431 properties in a subset of neurons. Overall, our study reveals a previously uncharacterized
432 population of *Chrna5*-expressing subplate neurons in the prefrontal cortex that are exquisitely
433 sensitive to acetylcholine, with differential expression of several lynx prototoxin genes that allow
434 flexible tuning of their high-affinity nicotinic responses (**Fig 5**).

435 Discussion (1106 words)

436 With optophysiological approaches in *Chrna5*-Cre transgenic mice, we identified a subpopulation
437 of prefrontal *Chrna5*-expressing neurons with faster and stronger cholinergic activation and high-
438 affinity nicotinic responses. Transcriptomic analysis revealed these Chrna5+ cholinergic 'super-
439 responders' to express markers associated with subplate neurons, a developmentally-defined
440 population associated with cortical layer 6b in adult rodents. These neurons show specific
441 expression pattern of several GPI-anchored lynx prototoxins known to modulate nicotinic receptor
442 function. Finally, by experimentally perturbing endogenous GPI-anchored lynx and exogenous
443 application of a cell-type specific lynx, we revealed complex lynx-mediated control of endogenous
444 nicotinic receptor function in prefrontal deep-layer neurons.

445 ***Specialized cholinergic properties of Chrna5-expressing neurons***
446 The functional impact of the auxiliary alpha5 nicotinic subunit in its native neuronal environment
447 is unclear. Previous work extrapolated the neurophysiological contributions of alpha5 to high-
448 affinity nicotinic receptors based on results of cell system experiments and work in rodents deleted
449 for *Chrna5* (*13*, *16*, *22*, *65*, *66*). Here, *Chrna5*-Cre mice allowed us to affirmatively demonstrate
450 that neurons expressing the alpha5 nicotinic subunit respond faster and more strongly to
451 endogenous acetylcholine. This cholinergic heterogeneity among layer 6 neurons prompted a
452 larger scale comparison between Chrna5+ neurons and a well-defined layer 6 population labeled
453 by *Syt6* (*29*). These experiments revealed a subset of Chrna5+ cholinergic 'super-responders' with
454 high affinity nicotinic responses that were not found in Syt6+ neurons.

455 ***Heterogeneity of cell types expressing Chrna5***
456 Previously, the deep-layer cell types expressing *Chrna5* were uncharacterized, and generally
457 thought to include L6CT neurons (*8*). Investigation of L6CT neurons have relied on *Syt6*-Cre and
458 *Ntsr1*-Cre mouse lines that label similar sets of neurons (*27–29*), with only *Syt*6-Cre mice
459 successfully labeling this population in prefrontal cortex (*31*). L6CT neurons act to control
460 attention through their thalamic projections (*67*, *68*), as well as their intracortical excitatory and
461 inhibitory outputs (*69–71*). L6CT neurons labeled by *Syt6* or *Ntsr1* expression are excited by
462 acetylcholine (*32*, *72*), but it was unclear what proportion of these cells were modulated by the α5
463 nicotinic subunit and the degree to which their nicotinic response relied on *Chrna5* expression.
464 Strikingly, our experiments reveal that the cholinergic super-responders with high affinity
465 nicotinic receptors appear to be from the population of *Chrna5*-labeled neurons that lack *Syt6*-
466 labeling. Our transcriptomic analysis demonstrates that majority of this likely population of
467 Chrna5+ 'super-responders' arise from L5 Near-Projecting and L6b neurons, populations that
468 express multiple markers that link them to the developmental subplate. These enigmatic cells are
469 remnants of the earliest-born cortical neurons that serve as a relay for establishing connections
470 between cortex and thalamus (*73*, *74*). Cholinergic innervation begins early in development and

subplate neurons already receive cholinergic inputs at birth (*75*), highlighting their role in developmental cholinergic modulation.

Chrna5- a marker for subplate cells

In contrast to L6CT neurons, subplate neurons are relatively uncharacterized due to the lack of transgenic mice that definitively label all subtypes as well as the inaccessibility of the available lines for *in vivo* targeting. We found Chrna5+ cholinergic super-responders predominantly arise from neurons that are not labeled by Syt6. Transcriptomic analysis (Fig 5, Table 1) suggests that this Chrna5+ population is enriched for known subplate markers *Ctgf* (Connective tissue growth factor), *Cplx3* (Complexin 3), *Kcnab1,*and *Lpar1* (*35*, *76*, *77*). Significantly, the lynx prototoxin and nicotinic receptor modulator *Ly6g6e*, which is highly expressed in Chrna5+ neurons, is also a marker of subplate neurons (*78*). Our study is the first to identify enhanced cholinergic activation regulated by *Chrna5* and lynx-gene expression in subplate/L6b neurons. Subplate neurons have recently been found to strongly regulate cortical output through their intracortical connections (*79*, *80*). Enhanced cholinergic activation in these neurons will have different consequences for prefrontal processing, challenging the popular conception that cholinergic modulation of attention occurs only through top-down control of thalamic input by L6CT neurons.

Molecular determinants of nicotinic receptor properties in Chrna5+ neurons

Our transcriptomic analysis revealed enhanced expression of GPI-anchored lynx prototoxin genes *Ly6g6e*, *Lypd1*, and *Lypd6b* in Chrna5+ neurons (Fig 5). Lynx proteins are well known modulators of nicotinic receptor properties and trafficking (*41*, *43*), but most of the insight into their actions comes from heterologous cell systems, deletion, and overexpression experiments. Relatively little is known about their effects on nicotinic receptors in their native environment. In expression systems, Ly6g6e potentiates α4β2 nicotinic responses and slows their desensitization (*39*), predicting cholinergic responses in Chrna5+ neurons would be resistant to desensitization, as has been implied by *Chrna5* deletion work (*16*). On the other hand, Lynx2 is a negative nicotinic modulator that increases desensitization of α4β2 nicotinic receptors (*61*), and is selectively expressed in the medial PFC (*81*). While Lynx2 is thought to act intracellularly to reduce surface expression of α4β2 nicotinic receptors (*39*), it may act selectively on the $(\alpha4)_3(\beta2)_2$ receptor subtype which are lower affinity (*65*, *82*) and indirectly promote expression of high affinity α5-containing $(\alpha4)_2(\beta2)_2\alpha5$ nicotinic receptors. The effect of Lypd6b on $(\alpha4)_2(\beta2)_2\alpha5$ nicotinic receptors found in Chrna5+ neurons is yet to be determined and may further contribute to the complex control of their nicotinic responses (*60*). In addition, Lynx1, a well known negative modulator of α4β2 nicotinic receptors (*42*, *83–85*) is also expressed uniformly across all Chrna5+ and Syt6+ neurons. Consistent with such complex regulation by lynxes, our experiments confirmed that removing GPI-anchored lynxes increases nicotinic response onset speed and amplitude in layer 6 neurons, potentially due to removal of Lynx1. In contrast, exogenous application of recombinant Ly6g6e protein had different effects in *Chrna5* and *Syt6*-expressing neurons, consistent with the differential lynx modulation in Chrna5+ neurons predicted by transcriptomics.

512 ***Functional consequences***

513 The effects of lynxes on nicotinic receptor function have so far been determined by heterologous
514 expression systems (*39*, *61*), knockout studies (*61*, *81*), exogenous application of recombinant
515 water-soluble lynx proteins (*86*, *87*), and more recently viral overexpression in the brain (*42*, *88*).
516 Our results are highlight complex endogenous regulation of optogenetic nicotinic responses by
517 multiple GPI-anchored lynxes. Expression of inhibitory lynxes and high levels of
518 acetylcholinesterase in Chrna5+ neurons suggest that their responses are restrained and our
519 experiments likely underestimated their nicotinic receptor function. These responses could be
520 dramatically enhanced when acetylcholinesterase and inhibitory lynx modulation is reduced
521 through other signaling mechanisms. Such flexible tuning of nicotinic responses by lynx
522 prototoxins in Chrna5+ neurons can provide greater dynamic range and poises them to be key
523 players during attentional processing (Fig 9). A recent study found that developmental increase in
524 Lynx1 expression in corticocortical neurons suppressed their nicotinic responses and preventing
525 this by viral knockdown of Lynx1 led to altered cortical connectivity and impaired attention (*42*).
526 Thus cell-type specific changes in lynx expression during development are critical for maturation
527 of attention circuits. It is of interest to examine such changes during development in Chrna5+
528 neurons and how they differ from Syt6+ neurons.

529 Our study reveals a distinct group of 'acetylcholine super-responder' neurons in the prefrontal
530 cortex identified by *Chrna5*-expression that include subplate neurons vital for cortical
531 development. We identify that their high affinity α5 subunit-containing nicotinic receptors are
532 under complex regulation by several lynx prototoxins and acetylcholinesterase. *Chrna5*-Cre mice
533 are a valuable tool for future studies examining the in vivo role of these specialized neurons.

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741 Corticothalamic Circuit for Dynamic Switching between Feature Detection and Discrimination.
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806 **Figure legends**

807 **Figure 1. Chrna5 expression identifies a distinct population of prefrontal neurons with**
808 **stronger and faster-onset optogenetic cholinergic responses. A,** Breeding scheme to obtain
809 triple transgenic *Chrna5*-Cre$^{/+}$Ai14$^{/+}$ChAT-ChR2$^{/+}$ mice expressing tdTomato in *Chrna5*-
810 expressing (Chrna5+) neurons and Channelrhodopsin 2 in cholinergic axons. **B,** Top- Schematic
811 of coronal mPFC slice with region of interest, adapted from (*89*). Bottom- two photon imaging (3D
812 projection) of tdTomato-labeled Chrna5+ neurons and EYFP-labeled cholinergic axons in layer 6
813 of mPFC slices. **C,** IRDIC (left) and widefield fluorescence (TRITC, right) images of tdTomato
814 labeled Chrna5+ and unlabeled layer 6 neurons during whole cell patch clamp electrophysiology.
815 Clearing induced by the pipette is visible. **D,** Average light-evoked endogenous cholinergic
816 response of labeled Chrna5+ vs neighbouring unlabeled Chrna5- neurons. Dotted lines are the
817 slope of the response onset. (Inset) Individual responses are zoomed in to show the onset. **E-F,** Bar
818 graph comparing (E) Rising slope and (F) Peak current of endogenous cholinergic responses
819 between labeled Chrna5+ neurons (n = 24 cells) and unlabeled Chrna5- neurons (n = 15 cells, 4
820 mice). $*P < 0.05$, Unpaired t-test. Top) **G,** (Top) Breeding scheme to obtain triple transgenic
821 *Chrna5*-Cre$^{/+}$Ai14$^{/+}$*Syt6*-EGFP$^{/+}$ mice expressing tdTomato in Chrna5+ neurons and EGFP in
822 Syt6+ neurons (Bottom) Confocal imaging in mPFC slices shows Chrna5+ and Syt6+ neurons
823 distributed in layer 6. **H-I,** Confocal (H) and two-photon imaging (I) reveal three populations of
824 neurons: exclusively Chrna5+ neurons which do not express *Syt6*, overlapping Chrna5+Syt6+
825 neurons which express both markers, and exclusively Syt6+ neurons which do not express *Chrna5*.
826 **J,** Left- Graph quantifies the percentage of each cell type with respect to all labeled cells per
827 sample. Right- Average proportions of Chrna5+, Chrna5+Syt6+ and Syt6+ neurons.

828 **Figure 2. Calcium imaging in Chrna5+ and Syt6+ populations reveals a distinct subset of**
829 **Chrna5+ neurons with resilient nicotinic responses. A,** Top: Two photon calcium imaging in
830 prefrontal brain slices from *Chrna5*-Cre$^{/+}$Ai96$^{/+}$ (left) and *Syt6*-Cre$^{/+}$Ai96$^{/+}$ mice (right) showing
831 acetylcholine-evoked GCaMP6s responses in Chrna5+ and Syt6+ neurons respectively (scale 50
832 μm). Bottom: Acetylcholine-evoked GCaMP6s signals were sequentially recorded after
833 application of competitive nicotinic antagonist DHBE and addition of muscarinic antagonist
834 atropine (scale 10 μm). **B,** Normalized fluorescence signal **(**ΔF by F) evoked by acetylcholine in
835 individual Chrna5+ and Syt6+ neurons in a brain slice at baseline (left), after DHBE (middle), and
836 after DHBE + Atropine (right). (Inset, average response and standard deviation. Scale: same as
837 main figure). **C-D i,** Boxplot shows the percentage of response remaining after the application of
838 (C) DHBE and (D) DHBE + Atropine (Inset shows the same boxplot with a restricted y-axis). +
839 denote respective means. Responses were quantified by the area under the ΔF/F curve (n = 71
840 neurons, 6 mice for Chrna5+, and 112 neurons, 7 mice for Syt6+, $****P < 10^{-4}$, Mann-Whitney
841 test). **C-D ii,** Cumulative frequency distribution of the percentage response remaining after **(**C)
842 DHBE and (D) DHBE + Atropine ($P < 10^{-4}$, Kolmogorov-Smirnov test). **C-D iii,** Proportion of
843 cells showing zero and non-zero responses after (C) DHBE and (D) DHBE + Atropine ($P < 10^{-4}$
844 for both C & D, Fisher's exact test). **E,** Current clamp responses evoked by 1mM acetylcholine
845 (15s) in fluorescently labeled Chrna5+ and Syt6+ layer 6 neurons patched in mPFC slices from
846 mice *Chrna5*-Cre$^{/+}$ Ai14$^{/+}$ and *Syt6*-Cre$^{/+}$Ai14$^{/+}$ or *Syt6*-EGFP mice respectively. **F,** Peak spike
847 frequency in Chrna5+ and Syt6+ neurons evoked by acetylcholine (left), in the presence of

848 competitive nicotinic antagonist DHBE and atropine (middle). Residual response remaining after
849 DHBE + Atropine in a distinct subset of Chrna5+ neurons is blocked by non-competitive nicotinic
850 antagonist mecamylamine (right). (**$P < 0.01$, *$P < 0.05$, unpaired t-test).

851 **Figure 3. Single cell transcriptomic analysis reveals Chrna5+ subset to span subplate neuron**
852 **populations with differential expression of lynx prototoxin genes A,** Single cell-RNAseq data
853 for 2422 L5-6 glutamatergic neurons in the anterior cingulate cortex (ACA, shown in schematic
854 on the left) was obtained from publicly available datasets (Allen Institute, SMARTSeq ACA and
855 MOP (2018)). Right- Scatter plot showing *Chrna5* vs *Syt6* expression in $\log_{10}$ (Copies per million)
856 for each neuron, with the frequency distribution shown on the corresponding axes. Neurons were
857 classified into Chrna5+, Chrna5+Syt6+ and Syt6+ groups based on their expression of *Chrna5* and
858 *Syt6* genes. Cells which expressed neither gene were excluded from subsequent analyses. **B**, The
859 major neuronal subclasses within Chrna5+, Chrna5+Syt6+ and Syt6+ groups is indicated by the
860 colorbar on top. NP- Near projecting, CT- Corticothalamic. Heatmap shows expression of
861 subplate and corticothalamic marker genes in each cell in all 3 groups. **C,** Dotplot shows summary
862 of subplate and corticothalamic marker expression in Chrna5+, Chrna5+Syt6+ and Syt6+ groups.
863 Dot size indicates the percentage of cells within each group expressing that gene, color of the dot
864 indicates average expression level relative to other groups. Chrna5+ neurons highly express
865 multiple subplate marker genes, but not corticothalamic markers. **D**, Violin plots show expression
866 of Lynx prototoxins *Ly6g6e*, *Lypd1* (Lynx2) and *Lypd6b* which show highest fold-change between
867 Chrna5+ and Chrna5+Syt6+ neurons. **E,** Dotplot shows expression of major genes known to
868 modulate cholinergic function, including nicotinic, muscarinic subunits, acetylcholinesterase, and
869 lynx prototoxins in Chrna5+, Chrna5+Syt6+ and Syt6+ neurons. Genes are ordered by decreasing
870 fold change in expression. Dot size indicates the percentage of cells within each group expressing
871 that gene, color of the dot indicates average expression level relative to other groups. Fold change
872 of all the genes shown in this dotplot are listed in Supplemetary table 3

873 **Figure 4. Regulation of optogenetic nicotinic responses by endogenous GPI-anchored lynxes**
874 **and cell type specific effects of recombinant Ly6g6e. A,** Schematic of nicotinic receptor
875 environment showing endogenous GPI-anchored lynxes exerting positive and negative modulation
876 of nicotinic receptors. The compound m-3M3FBS activates PLC, cleaving the GPI anchor and
877 perturbing lynx-mediated modulation of nicotinic responses. **B,** Optogenetic nicotinic responses
878 in prefrontal deep-layer pyramidal neurons from ChAT-ChR2 mice before and after treatment with
879 m-3M3FBS (5 min). **C,** PLC activation significantly increased the rising slope of optogenetic
880 nicotinic responses. Change in **D,** Rising slope and **E,** Area of nicotinic response in control and
881 after PLC activation. (*$P < 0.05$, **$P < 0.01$, Wilcoxon matched-pairs test). **E,** IRDIC (left) and
882 widefield fluorescence (right) images of tdTomato labeled Chrna5+ (top) and Syt6+ (bottom) deep
883 layer neurons during whole-cell patch clamp electrophysiology in *Chrna5*-Cre$^{/+}$Ai14$^{/+}$ChAT-
884 ChR2$^{/+}$ and *Syt6*-Cre$^{/+}$Ai14$^{/+}$ChAT-ChR2$^{/+}$ mouse brain slices respectively. **G,** Schematic
885 summarising predicted and observed effects of recombinant water-soluble ly6g6e on Chrna5+ and
886 Syt6+ neuronal nicotinic receptors. **G,** Optogenetic nicotinic responses are reduced in amplitude
887 following 10 minute application of soluble ly6g6e in Syt6+ but not Chrna5+ neurons. Change in
888 peak current (I) and area of the nicotinic response (**J**) of Chrna5+ vs Syt6+ neurons (*$P < 0.05$, **
889 $P < 0.01$, Unpaired t-test)

890 **Figure 5. Graphical summary.** Deep-layer pyramidal neurons can be divided into three groups
891 (Chrna5+, Chrna5+Syt6+, Syt6+) by their expression of *Chrna5* and *Syt6* genes. The subset of
892 *Chrna5*-expressing neurons without *Syt6* expression are molecularly distinct and comprise of
893 subplate neurons, whereas *Syt6*-expressing neurons are of the corticothalamic subtype. Nicotinic
894 receptors in these neurons are under complex regulation by endogenous lynx prototoxins.
895 Inhibitory prototoxin gene *Lynx1* is expressed uniformly in all neurons, whereas Chrna5+ subplate
896 neurons additionally have specific expression of *Ly6g6e*, *Lypd1* and *Lypd6b* prototoxin genes.
897 These Chrna5+ subplate neurons show enhanced α5 subunit nicotinic receptor-mediated
898 cholinergic responses that are differently modulated by specific lynx prototoxins.

899 **Supplementary materials**

900 **Supplementary video 1 & 2:** Videos show **(1)** Chrna5+ neurons in mPFC slices from *Chrna5*-
901 Cre$^{/+}$Ai96$^{/+}$ mice and **(2)** Syt6+ neurons from *Syt6*-Cre$^{/+}$Ai96$^{/+}$ mice responding to exogenous
902 application of 1mM acetylcholine with an increase in GCaMP6s fluorescence signal.

903 **Supplementary table 1:** Intrinsic electrophysiological properties of Chrna5+ and Chrna5-
904 unlabeled deep-layer neurons in *Chrna5*-Cre$^{/+}$Ai14$^{/+}$ChAT-ChR2$^{/+}$ mice.

905 **Supplementary table 2:** Intrinsic electrophysiological properties of Chrna5+ and Syt6+ deep-
906 layer neurons.

907 **Supplementary table 3:** Table comparing expression of major genes modulating postsynaptic
908 cholinergic responses in Chrna5+ and Chrna5+Syt6+ neurons.

909 **Supplementary table 4.** Table of all differentially expressed genes between Chrna5+ and
910 Chrna5+Syt6+ neurons with adjusted p value < 0.05

Figures and Tables

Figure 1

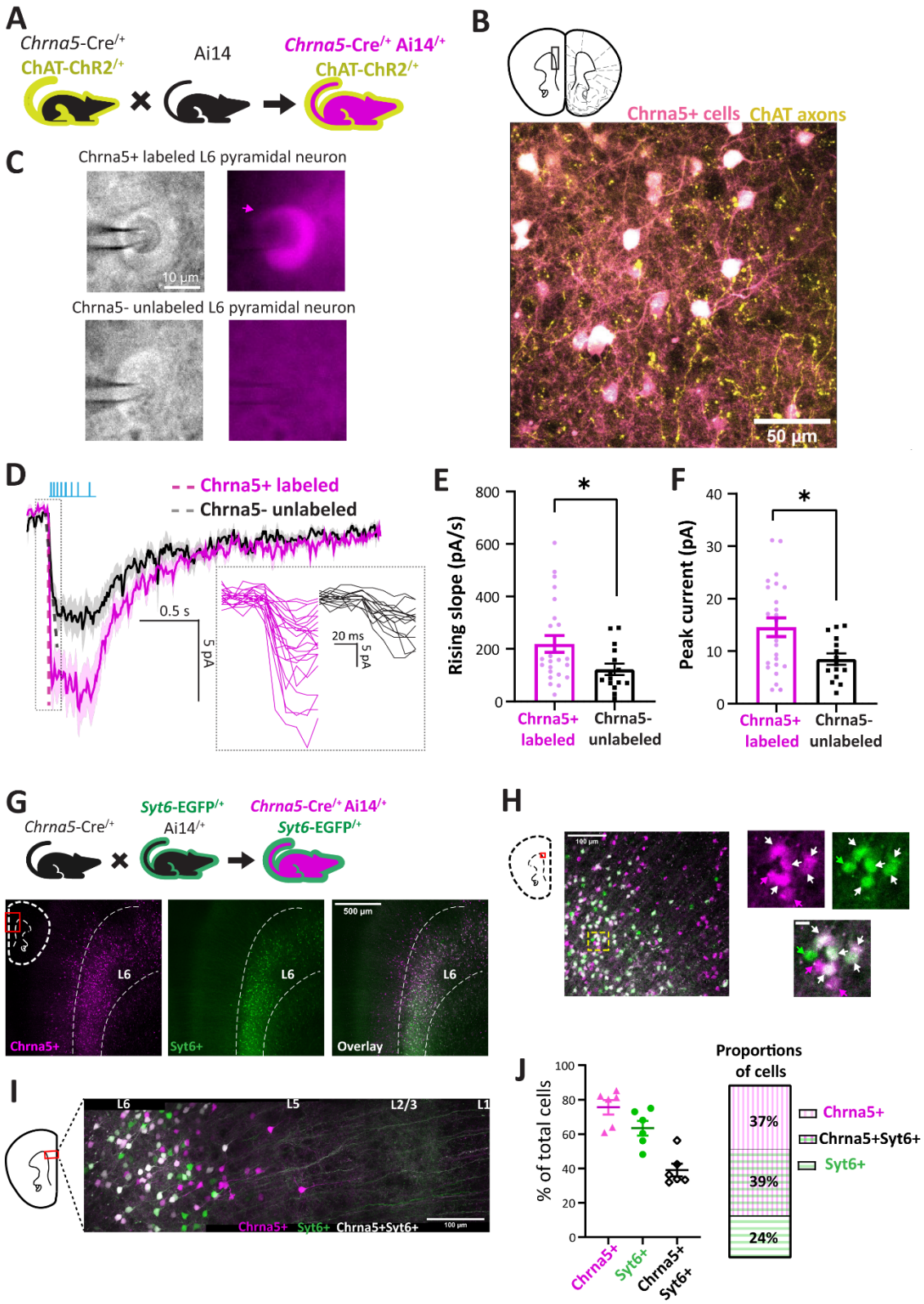

Figure 2

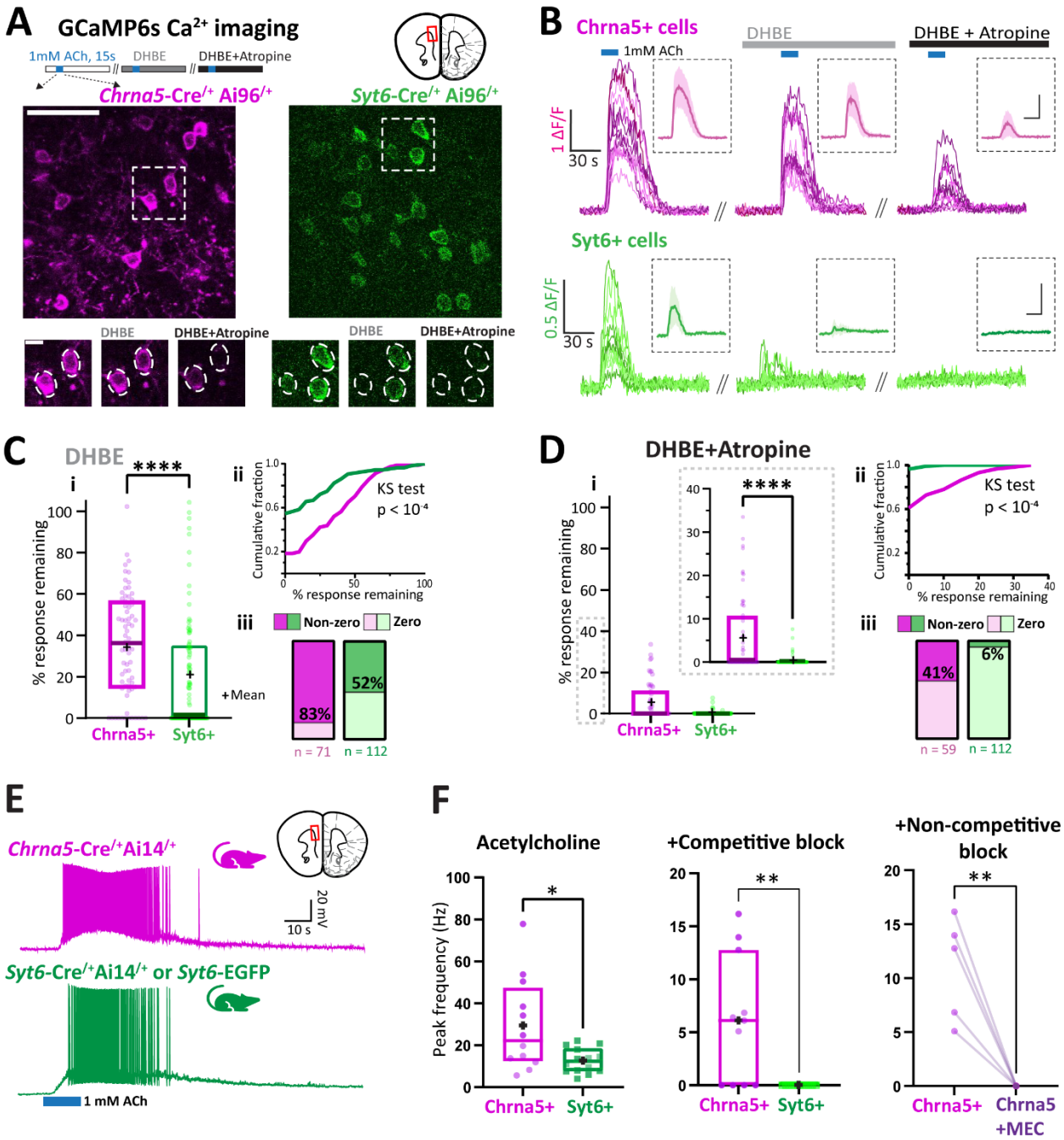
A
GCaMP6s Ca2+ imaging
1mM ACh, 15s
DHBE
DHBE+Atropine
Chrna5-Cre/+ Ai96/+
Syt6-Cre/+ Ai96/+
B
Chrna5+ cells
1mM ACh
DHBE
DHBE + Atropine
1 ΔF/F
30 s
Syt6+ cells
0.5 ΔF/F
C
DHBE
****
% response remaining
Chrna5+
Syt6+
+Mean
KS test
p < 10-4
Cumulative fraction
Non-zero
Zero
83%
52%
n = 71
n = 112
D
DHBE+Atropine
41%
6%
n = 59
n = 112
E
Chrna5-Cre/+Ai14/+
Syt6-Cre/+Ai14/+ or Syt6-EGFP
20 mV
10 s
1 mM ACh
F
Acetylcholine
+Competitive block
+Non-competitive block
Peak frequency (Hz)
Chrna5 +MEC

Figure 3

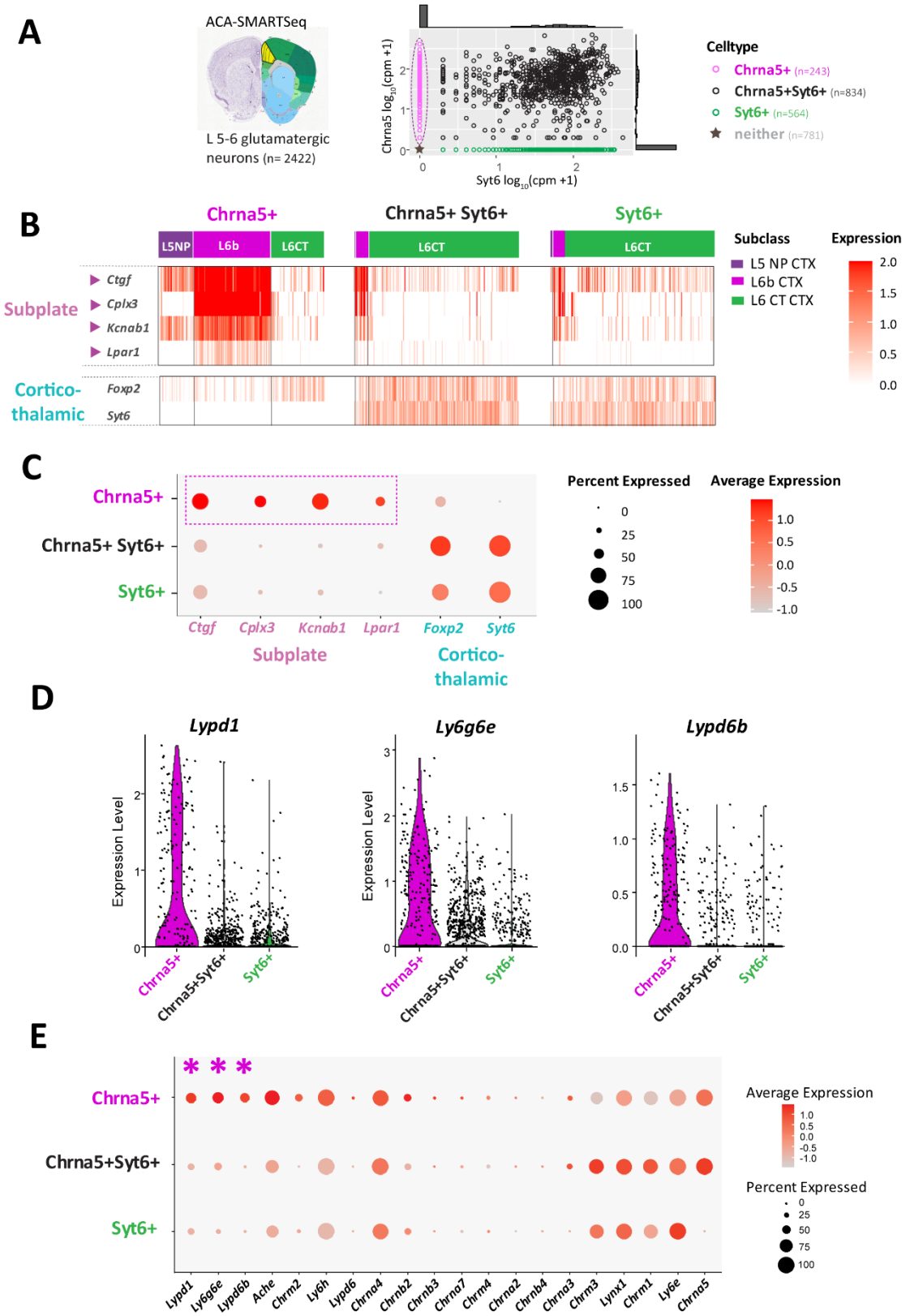
A
ACA-SMARTSeq
L 5-6 glutamatergic neurons (n= 2422)
Chrna5 log10(cpm +1)
Syt6 log10(cpm +1)
Celltype
Chrna5+ (n=243)
Chrna5+Syt6+ (n=834)
Syt6+ (n=564)
neither (n=781)
B
Chrna5+
Chrna5+ Syt6+
Syt6+
L5NP
L6b
L6CT
Subclass
L5 NP CTX
L6b CTX
L6 CT CTX
Expression
Subplate
Ctgf
Cplx3
Kcnab1
Lpar1
Cortico-thalamic
Foxp2
Syt6
C
Chrna5+
Chrna5+ Syt6+
Syt6+
Ctgf
Cplx3
Kcnab1
Lpar1
Foxp2
Syt6
Subplate
Cortico-thalamic
Percent Expressed
Average Expression
D
Lypd1
Ly6g6e
Lypd6b
Expression Level
Chrna5+
Chrna5+Syt6+
Syt6+
E
Chrna5+
Chrna5+Syt6+
Syt6+
Lypd1
Ly6g6e
Lypd6b
Ache
Chrm2
Ly6h
Lypd6
Chrna4
Chrnb2
Chrnb3
Chrna7
Chrm4
Chrna2
Chrnb4
Chrna3
Chrm3
Lynx1
Chrm1
Ly6e
Chrna5
Average Expression
Percent Expressed

Figure 4

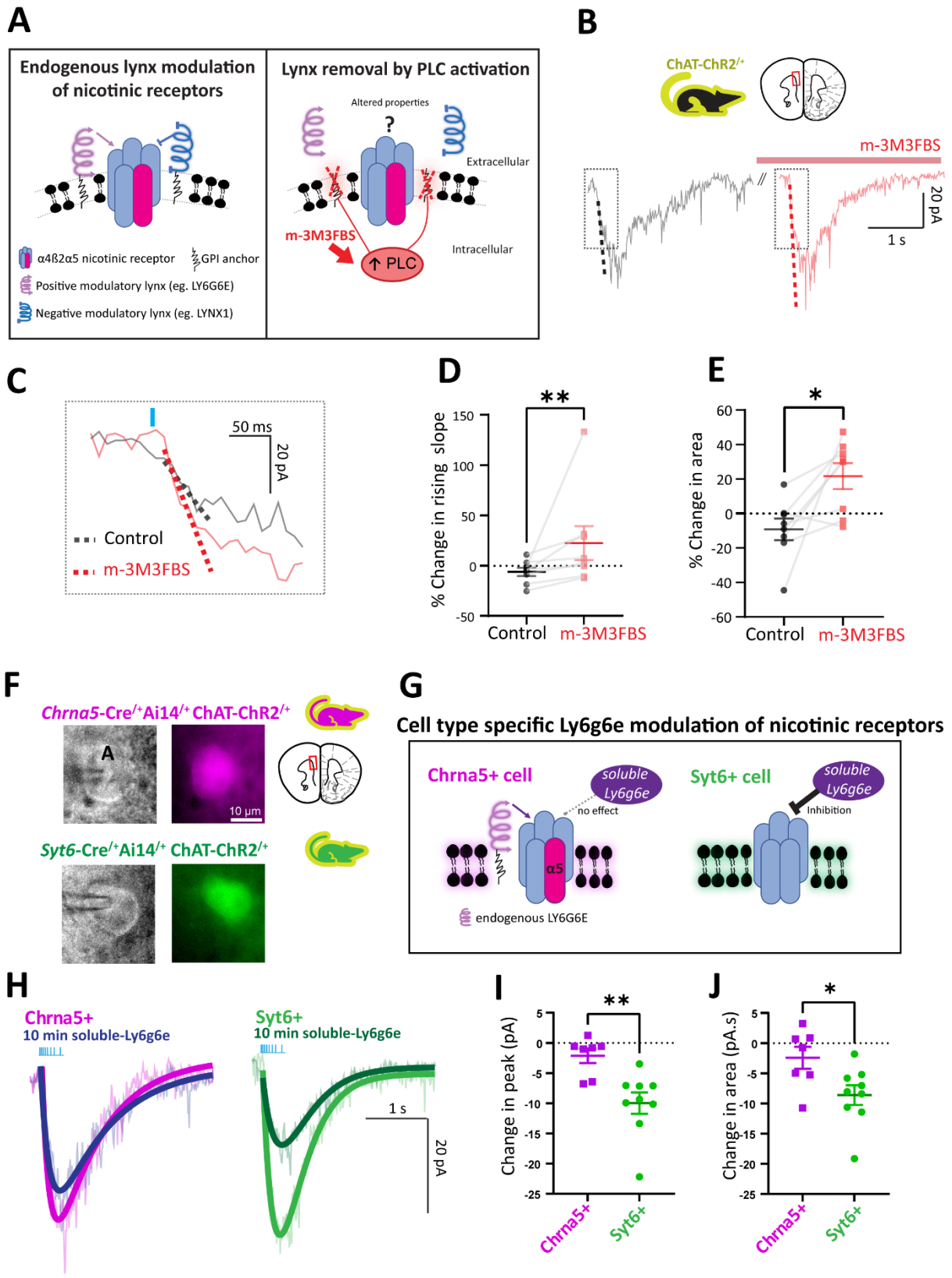
A
Endogenous lynx modulation of nicotinic receptors
α4ß2α5 nicotinic receptor
GPI anchor
Positive modulatory lynx (eg. LY6G6E)
Negative modulatory lynx (eg. LYNX1)
Lynx removal by PLC activation
Altered properties
?
Extracellular
m-3M3FBS
↑ PLC
Intracellular
B
ChAT-ChR2/+
m-3M3FBS
20 pA
1 s
C
50 ms
20 pA
Control
m-3M3FBS
D
**
% Change in rising slope
150
100
50
0
-50
Control
m-3M3FBS
E
*
% Change in area
60
40
20
0
-20
-40
-60
Control
m-3M3FBS
F
Chrna5-Cre/+Ai14/+ ChAT-ChR2/+
A
10 µm
Syt6-Cre/+Ai14/+ ChAT-ChR2/+
G
Cell type specific Ly6g6e modulation of nicotinic receptors
Chrna5+ cell
soluble Ly6g6e
no effect
α5
endogenous LY6G6E
Syt6+ cell
soluble Ly6g6e
Inhibition
H
Chrna5+
10 min soluble-Ly6g6e
Syt6+
10 min soluble-Ly6g6e
1 s
20 pA
I
**
Change in peak (pA)
5
0
-5
-10
-15
-20
-25
Chrna5+
Syt6+
J
*
Change in area (pA.s)
5
0
-5
-10
-15
-20
-25
Chrna5+
Syt6+

Figure 5

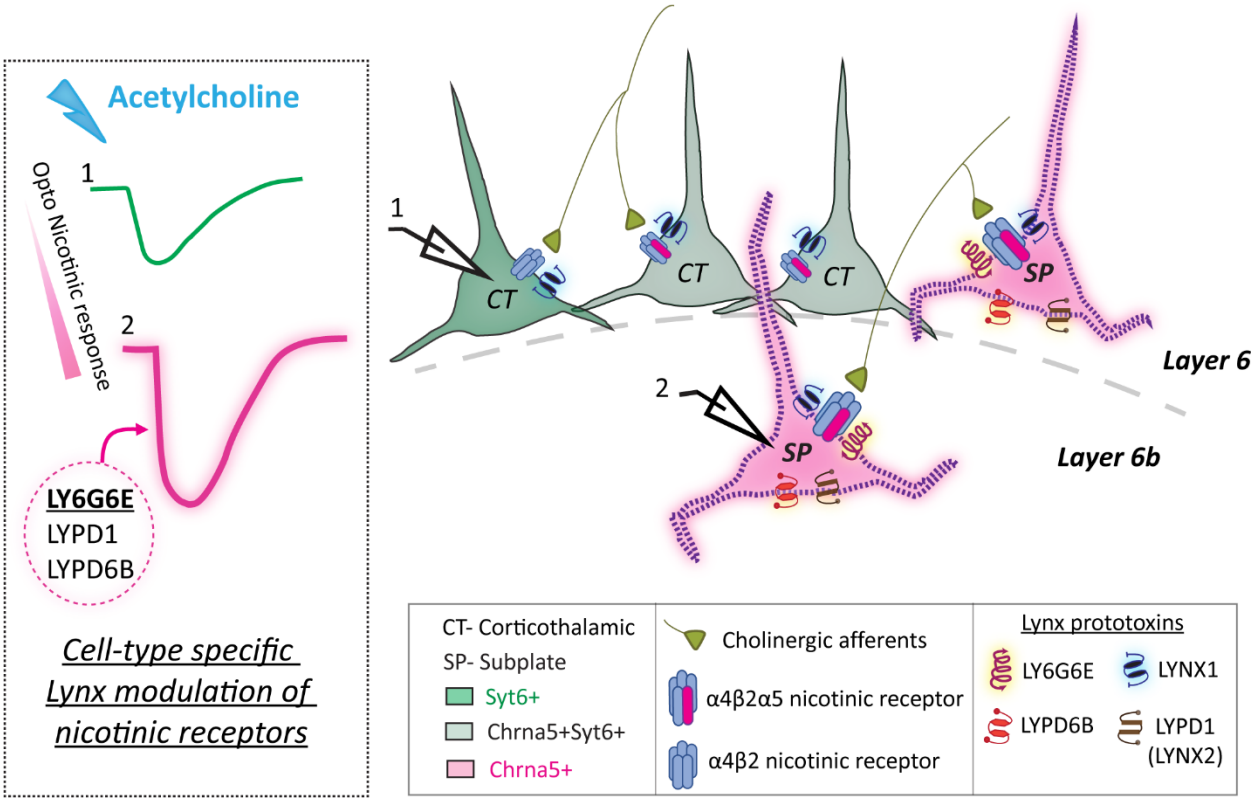
Acetylcholine
Opto Nicotinic response
1
2
LY6G6E
LYPD1
LYPD6B
Cell-type specific Lynx modulation of nicotinic receptors
CT
SP
Layer 6
Layer 6b
CT- Corticothalamic
SP- Subplate
Syt6+
Chrna5+Syt6+
Chrna5+
Cholinergic afferents
α4β2α5 nicotinic receptor
α4β2 nicotinic receptor
Lynx prototoxins
LY6G6E
LYNX1
LYPD6B
LYPD1 (LYNX2)

Table 1

Genes	P value	Chrna5+ cells %	Chrna5+Syt6+ cells %	Adjusted P value	Fold change (Chrna5+/ Chrna5+Syt6)
Cplx3	1.16E-43	0.535	0.149	3.37E-39	5.69
Ctgf	1.60E-28	0.77	0.568	4.65E-24	3.81
Ptn	3.68E-28	0.864	0.758	1.07E-23	3.60
Tmem163	2.12E-44	0.51	0.129	6.16E-40	2.58
Lypd1	3.40E-23	0.593	0.381	9.88E-19	2.55
Kcnab1	3.81E-74	0.741	0.191	1.11E-69	2.51
Serpini1	1.54E-50	1	0.993	4.49E-46	2.50
Hpcal1	2.48E-27	0.658	0.39	7.20E-23	2.47
Nrsn2	1.89E-30	0.864	0.767	5.50E-26	2.28
Ntm	1.83E-56	0.889	0.59	5.31E-52	2.21
Tshz2	2.82E-16	0.535	0.327	8.20E-12	2.17
Etv1	5.70E-16	0.395	0.186	1.66E-11	2.16
Rcn2	5.18E-46	0.996	0.984	1.51E-41	2.14
Olfm3	1.67E-44	0.897	0.716	4.86E-40	2.11
Crtac1	5.50E-46	0.728	0.347	1.60E-41	2.04
Ly6g6e	2.13E-25	0.671	0.434	6.20E-21	2.03
Adk	6.31E-29	0.922	0.922	1.83E-24	2.00
Trp53i11	2.73E-60	0.724	0.222	7.94E-56	1.93
Cd164	6.72E-18	0.897	0.882	1.95E-13	1.92
Dner	2.35E-45	0.712	0.326	6.82E-41	1.85

Top 20 differentially expressed genes between Chrna5+ and Chrna5+Syt6+ neuron. Top 20 differentially expressed genes determined by the FindMarkers function on Seurat ordered by decreasing fold change. Several known subplate neuron markers (highlighted by *****) are the highest enriched genes in Chrna5+ neurons. All differentially expressed genes are listed in supplementary table 4.